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Receptor activator of nuclear factor- κ B ligand (RANKL) directly modulates the gene expression profile of RANK-positive Saos-2 human osteosarcoma cells

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Abstract. Receptor activator of nuclear factor κ B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) are the key regulators of bone metabolism. Recent findings demonstrated a crucial role of RANK in several bone-associated tumors. Indeed, we have recently demonstrated functional RANK expression both in a mouse and several human osteosarcoma cell lines. However, RANKL effects on osteosarcoma cells remain to be determined. In this study, we determined RANKL effects on RANK-positive Saos-2 human osteosarcoma cells. cDNA microarray and quantitative RT-PCR analyses clearly demonstrated that RANK-positive osteosarcoma cells were the target of RANKL as well as osteoclasts/osteoclast precursors. Thus, we present for the first time that RANKL can directly and significantly modulate gene expression of RANK-expressing Saos-2 cells. RANKL-modulated genes included genes that were implicated in protein metabolism, nucleic acid metabolism, intracellular transport, cytoskeleton organization and biogenesis, apoptosis and signaling cascade. Our results strengthen the involvement of the RANK/RANKL/OPG axis in osteosarcoma biology and capability to identify novel therapeutic approaches targeting RANK-positive osteosarcomas.

Introduction

Osteosarcoma is the most frequent malignant primary bone tumor. Some important pathogenetic roles of *p53*, *RB* and *mdm2* have been already reported (1); however the pathogenesis of osteosarcoma and mainly the role of the bone microenvironment in cancer cell biology are not fully understood. Therefore, understanding the biological mechanisms that govern osteosarcoma development at the molecular level should lead to the determination of new potential therapeutic targets.

The discovery of key factors involved in the control of osteoclastogenesis has moved bone research into a new era. The most notable of these factors belong to the tumor necrosis factor (TNF)/TNF receptor family: receptor activator of nuclear factor κ B (RANK/TNFRSF11A), its ligand RANKL/TNFSF11 and decoy receptor for RANKL, osteoprotegerin (OPG/TNFRSF11B) (2-4). Consequently, RANKL has been shown both to mediate osteoclastogenesis and activate mature osteoclasts, whereas OPG negatively regulates RANKL binding to RANK, reduces the half-life of membranous RANKL, therefore inhibiting bone resorption induced by osteoclasts (5). RANK/RANKL/OPG axis is the key regulator of bone metabolism not only in normal but also pathological conditions. Indeed, bone-related tumors including osteosarcoma are very often associated with dysregulated RANK/RANKL/OPG axis leading to altered bone remodeling (6,7). RANK has also attracted special attention because a functional RANK expression has been reported in several bone-associated tumors (8-11). Interestingly, RANKL triggered migration of human prostate cancer cells (8,9), breast cancer cells and melanoma cells that express RANK (9). Recently, we have reported functional RANK expression in a mouse (POS-1 cells) (10) and several human osteosarcoma cell lines (11). All these findings suggest the major involvement of RANK/RANKL/OPG axis in osteosarcoma which appears to be one of the most relevant and confidential therapeutic targets.

In the present study, we analyzed the RANKL effect on RANK-positive human osteosarcoma cells using Saos-2 cells that expresses functional RANK.

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Key words: RANK, RANKL, osteosarcoma, bone tumor, bone microenvironment, gene modulation

Materials and methods

Cell culture. The human osteosarcoma cell line Saos-2 was purchased from the American Tissue Cell Collection (LGC Promochem, Molsheim, France). Saos-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Bio Whittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) (Hyclone-Perbio, Brebières, France) at 37°C in a humidified atmosphere (5% CO₂ and 95% air).

Cell proliferation and viability assays. Cell proliferation was determined as previously reported with slight modifications (8). Briefly, Saos-2 cells were seeded at the density of 2x10³ cells per well into a 96-multiwell plate, and cultured for 72 h in DMEM supplemented with 0.5% FCS in the absence or presence of recombinant human RANKL (rhRANKL) kindly provided by Amgen Inc. (Thousand Oaks, CA, USA) (5, 50, 100 ng/ml). The medium was changed every 24 h and the cell proliferation was determined by an XTT based method, using Cell Proliferation Kit II (Sigma, Saint-Quentin Fallavier, France) following to the supplier's recommendations. In addition, trypan-blue exclusion was used to quantify the viable and dead cells. Saos-2 cells were seeded into a 24-multiwell plate (5x10³ cells/well) and cultured in DMEM supplemented with several FCS concentrations (0.5-10%) in the absence or presence of 5 to 100 ng/ml rhRANKL, and then the viable and dead cell number was counted at days 1, 4 and 7 under a light microscope.

Cell migration analyses by slit assay. Cell migration analyses were performed as previously described with slight modifications (8). Briefly, Saos-2 cells were seeded at the density of 40x10³ cells per well into a 24-multiwell plate, and cultured in DMEM supplemented with 1% FCS. At the time of confluence, cells were incubated in the absence or presence of rhRANKL (5, 50 and 100 ng/ml) for 24 h. Then, a slit was made horizontally with a white tip at the centre of each confluent well, the medium was changed after gentle rinse and cells were cultured for 24 h with or without rhRANKL (5, 50 and 100 ng/ml). Cell invasion on the slit of the confluent well was assessed in each condition by light microscope.

cDNA microarray. Total RNAs were extracted using TRIzol reagent (Invitrogen, Eragny, France) from Saos-2 osteosarcoma cell line cultured in the absence (control condition, n=3) or presence of 50 ng/ml rhRANKL (treatment condition, n=3) for 24 h. One microgram of total RNA was amplified using the Amino Allyl MessageAmp™ II-aRNA amplification Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions: 2 amplifications were performed from each control condition (n=6), 3 amplifications from each treatment condition (n=9). Cy3- and Cy5-labeled Amino-allyl RNA (aaRNA) samples were prepared using the CyDye Post Labeling Reactive Dye Pack (Amersham BioSciences, Uppsala, Sweden). aaRNA samples from treatment condition were labeled with Cy3. The same amount of control aaRNA was pooled, labeled with Cy5 and used as the reference. For each step of the preparation, sample quality was assessed by capillary electrophoresis with Bioanalyser 2100 (Agilent

Technologies, Waldbronn, Germany). Each Cy3-labeled sample was mixed with an equal amount of Cy5-labeled reference and then hybridized to the microarrays. Microarrays were prepared by INSERM U533 Laboratory (Dr J. Léger, Nantes, France) using 50-mer oligonucleotide probes (MWG Biotech, Hebersberg, Germany). The 6864 genes, spotted in triplicate on the microarrays, were composed of relevant gene collections already selected by teams from the West of France specialized in studies of various types of cancer or their related mechanisms (for further information see: http://cardioserve.nantes.inserm.fr/ptf-puce/cancerochips_en.php) (12). Hybridized arrays were scanned at 10-μm resolution with a Scanarray 3000 (Packard Biosciences, Wellesley). Data were analyzed with GenePix Pro 4.0 (Axon Union City, CA, USA). Raw data were normalized with the Madsan application (12,13) according to the instructions. Genes lacking valid expression values for more than one array were eliminated for all conditions. Two-class impaired Significance Analysis of Microarrays (SAM) was used to identify genes with statistically significant differential expression between control and treatment conditions (14).

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR (qRT-PCR) was performed in the Stratagene Mx3000p (Stratagene, CA, USA) using SYBR Green I dye and Sure Start Taq Polymerase (Stratagene), according to the manufacture's recommendations. The primers were designed with Beacon Designer 5.0 (Premier Biosoft). Validation of primers was done by optimization of each couple of primers between 50-900 nM each. Fluorescence was measured at the end of extension period by ramping from 60 to 95°C (0.2°C step) to generate a melting curve for each set of primers. The lowest C_t found for a set of primer was used the determination in qPCR and checked in a 2% agarose gel. qPCRs were performed with the 'Comparative qPCR' program of the MxPro software (Stratagene). Reaction was achieved with 1X Brilliant[®] SYBER[®]Green Master Mix (Stratagene), appropriated volumes of each primer, 30 nM final concentration of SYBER[®]Green, 5 μl of cDNA diluted 1:20. Amplification and detection were performed using the Mx3000P system (Stratagene), with β-actin as normalizer and controls as calibrators. The following profile: 1 cycle of 95°C for 10 min and 40 cycles each of 95°C for 30 sec, 60°C for 1 min, and 72°C for 30 sec was applied for all experiments. Fluorescence was measured at the end of annealing period of each cycle to monitor the amplification and was plotted in real-time manner. qPCR analysis was automatically done by the software. Comparative quantification was calculated with the formula of Pfaffl *et al* (15). Discrepancies were corrected with serial dilutions generating a standard curve, then if an efficiency of amplification between 95 and 105% was found, the difference between the Genes of Interest (GOI) and the Normalizer (housekeeping) genes fold change results can be obtained using Comparative Quantification algorithms.

Statistical analyses. Mann-Whitney's U test was employed when appropriate. Results with p<0.05 were considered significant.

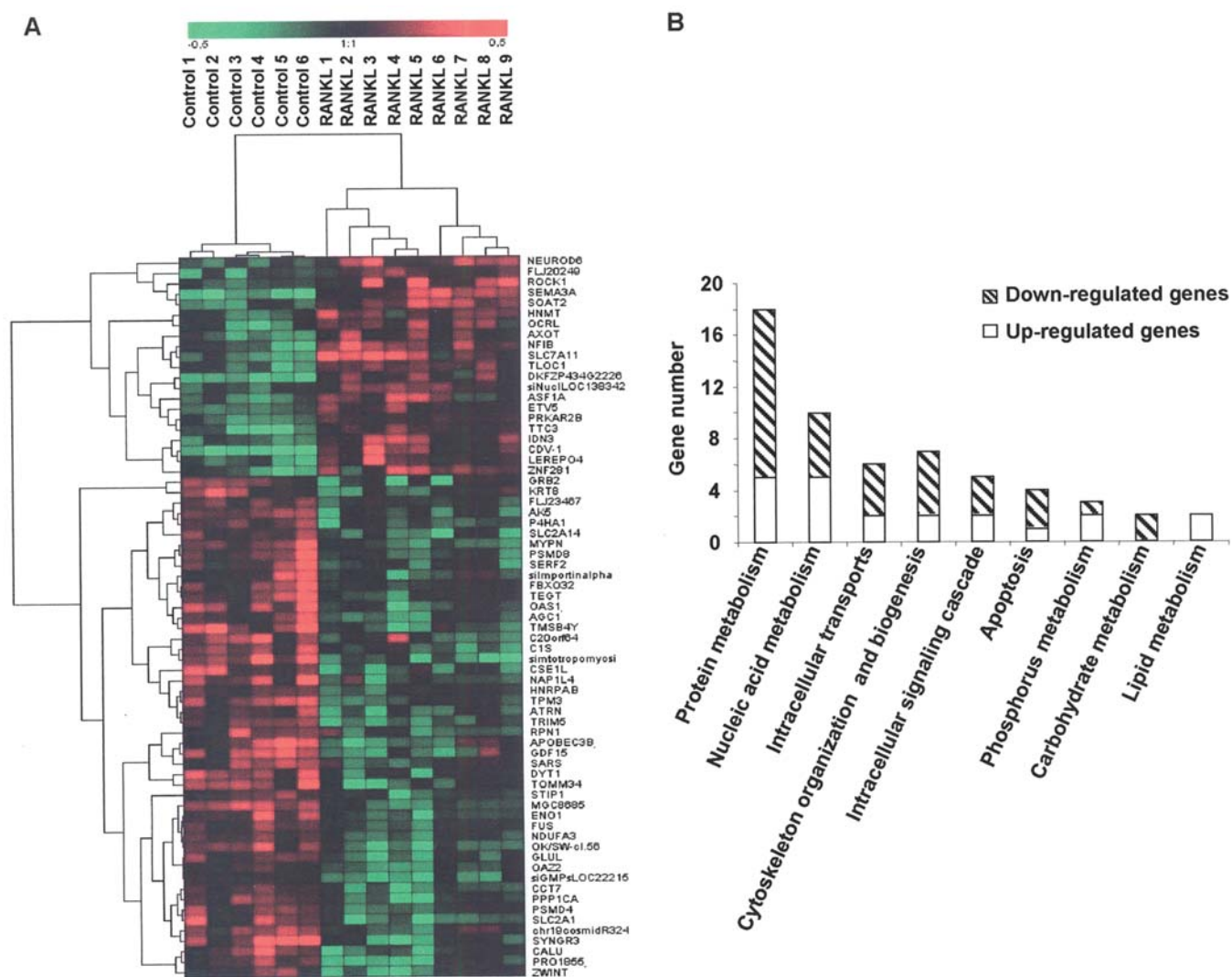


Figure 1. RANKL modulated the expression of 69 genes in human osteosarcoma Saos-2 cell line. (A) Two-way hierarchical clustering of the human osteosarcoma Saos-2 cell line and the 69 genes differentially expressed between control and RANKL-stimulated condition. The position of each is mentioned on the right. Expression values are indicated by color coding: red > grey > green. For each gene, the expression values were median centered and therefore represent relative expression ratios. (B) Gene Ontology classification of the genes differentially regulated by RANKL in Saos-2 cells. Representation of the genes up- and down-regulated by RANKL is according to the biological process categories (as defined by the Gene Ontology Consortium).

Results

Cell proliferation, viability and cell migration assays. Neither XTT assay nor manual cell counting could demonstrate any significant difference of Saos-2 cell proliferation and viability after rhRANKL treatment (data not shown). Moreover, rhRANKL did not modulate Saos-2 cell migration in the experimental conditions used (data not shown).

RANKL-induced gene modulations in RANK-positive Saos-2 cells. Three independent cDNA microarray experiments clearly demonstrated that RANKL was a powerful modulator of genes expressed by osteosarcoma cells (Fig. 1A). Thus, 69 genes out of 6,864 genes analyzed, showed significantly different levels of expression in rhRANKL-treated Saos-2 cells compared to the control group; 48 were down-regulated whereas the remaining 21 were up-regulated (Fig. 1B and Table I). The down-regulated group involved some genes implicated in protein

metabolism, nucleic acid metabolism, intracellular transport, cytoskeleton organization and biogenesis and apoptosis and signaling cascade. In the up-regulated group, the main genes affected by RANKL as referred to ontology biological processes were nucleic acid and protein metabolisms (Table I).

To confirm the results of cDNA microarray analysis, 10% of rhRANKL-modulated genes were measured by qRT-PCR. For instance, ROCK1 (Rho associated, coiled-coil containing protein kinase 1) and SEMA3A (Semaphorin 3A), significantly up-regulated by rhRANKL as revealed by microarray analysis (1.21- and 1.43-fold respectively) were also markedly up-regulated in qRT-PCR assay (2.59- and 17.1-fold, respectively, $p < 0.001$) (Fig. 2). Furthermore, the expression of GDF15 (growth differentiation factor 15) was down-regulated 0.78-fold in microarray analysis and around 0.8-fold in qRT-PCR ($p < 0.05$) (Fig. 2). Correspondingly, other results obtained through cDNA microarray analysis were also confirmed by qRT-PCR.

Table I. Detailed information of 69 genes expressed by human Saos-2 cell line modulated by RANKL.

Full name	Genebank accession no.	Gene symbol	Map location	Fold change
1 Aggrecan 1	M55172	<i>AGC1</i>	15q26.1	0.82
2 Adenylate kinase 5	BC036666	<i>AK5</i>	1p31	0.82
3 Apolipoprotein B mRNA editing enzyme	NM_004900	<i>APOBEC3B</i>	22q13.1-q13.2	0.76
4 Attractin	NM_139321	<i>ATRIN</i>	20p13	0.83
5 Complement component 1, s subcomponent	NM_001734	<i>C1S</i>	12p13	0.84
6 Chromosome 20 open reading frame 64	NM_033550	<i>C20orf64</i>	20q13.2	0.85
7 Calumenin	NM_001219	<i>CALU</i>	7q32	0.82
8 Chaperonin containing TCP1, subunit 7 (eta)	NM_006429	<i>CCT7</i>	2p13.2	0.85
9 Chromosome 19, cosmid R32469, complete sequence	AC005197	<i>chr19cosmidR324</i>	19p12	0.86
10 CSE1 chromosome segregation 1-like (yeast)	AF053640	<i>CSE1L</i>	20q13	0.81
11 Dystonia 1, torsion (autosomal dominant; torsin A)	AF007871	<i>DYT1</i>	9q34	0.81
12 Enolase 1, (alpha)	X84907	<i>ENO1</i>	1p36.3-p36.2	0.83
13 F-box only protein 32	NM_058229	<i>FBXO32</i>	8q24.13	0.86
14 Hypothetical protein FLJ23467	AF271774	<i>FLJ23467</i>	1q21.3	0.87
15 Fusion [involved in t(12;16) in malignant liposarcoma]	NM_004960	<i>FUS</i>	16p11.2	0.87
16 Growth differentiation factor 15	BC000529	<i>GDF15</i>	19p13.1-13.2	0.78
17 Glutamate-ammonia ligase (glutamine synthase)	BC031964	<i>GLUL</i>	1q31	0.83
18 Growth factor receptor-bound protein 2	NM_002086	<i>GRB2</i>	17q24-q25	0.82
19 Heterogeneous nuclear ribonucleoprotein A/B	NM_031266	<i>HNRPAB</i>	5q35.3	0.87
20 Keratin 8	NM_002273	<i>KRT8</i>	12q13	0.84
21 Tubulin, beta polypeptide paralog	BC001352	<i>MGC8685</i>	6p25	0.78
22 Myopalladin	AK027343	<i>MYPN</i>	10q22.1	0.80
23 Nucleosome assembly protein 1-like 4	NM_005969	<i>NAP1L4</i>	11p15.5	0.82
24 NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9 kDa	NM_004542	<i>NDUFA3</i>	19q13.42	0.86
25 2',5'-oligoadenylate synthetase 1, 40/46 kDa	NM_002534	<i>OAS1</i>	12q24.1	0.78
26 Ornithine decarboxylase antizyme 2	NM_002537	<i>OAZ2</i>	15q22.1	0.86
27 Beta 5-tubulin	NM_178014	<i>OK/SW-cl.56</i>	6p21.32	0.78
28 Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I	M24486	<i>P4HA1</i>	10q21.3-q23.1	0.85
29 Protein phosphatase 1, catalytic subunit, alpha isoform	NM_002708	<i>PPP1CA</i>	11q13	0.83
30 Hypothetical protein PRO1855	NM_018509	<i>PRO1855</i>	17q21.33	0.79
31 Proteasome 26S subunit, non-ATPase, 4	NM_002810	<i>PSMD4</i>	1q21.3	0.85
32 Proteasome 26S subunit, non-ATPase, 8	NM_002812	<i>PSMD8</i>	19q13.13	0.82
33 Ribophorin I	NM_002950	<i>RPN1</i>	3q21.3-q25.2	0.85
34 Seryl-tRNA synthetase	NM_006513	<i>SARS</i>	1p13.3-p13.1	0.86
35 Small EDRK-rich factor 2	NM_005770	<i>SERF2</i>	15q15.1	0.84
36 GMP synthase (glutamine-hydrolyzing), mRNA	XM_167338	<i>siGMPsLOC222152</i>	7	0.84
37 Importin alpha-2 subunit, mRNA	XM_070941	<i>siImportinalpha</i>	9	0.84
38 Similar to tropomyosin 4, mRNA	XM_088391	<i>simtotropomyosi</i>	8	0.74
39 Solute carrier family 2 (facilitated glucose transporter), member 1	K03195	<i>SLC2A1</i>	1p35-p31.3	0.77
40 Solute carrier family 2 (facilitated glucose transporter), member 14	AF481879	<i>SLC2A14</i>	12p13.31	0.81
41 Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	NM_006819	<i>STIP1</i>	11q13	0.86

Table I. Continued.

Full name	Genebank accession no.	Gene symbol	Map location	Fold change
42 Synaptogyrin 3	NM_004209	<i>SYNGR3</i>	16p13	0.74
43 Testis enhanced gene transcript (BAX inhibitor 1)	NM_003217	<i>TEGT</i>	12q12-q13	0.84
44 Thymosin, beta 4, Y-linked	NM_004202	<i>TMSB4Y</i>	Yq11.221	0.75
45 Translocase of outer mitochondrial membrane 34	NM_006809	<i>TOMM34</i>	20q12-q13.1	0.77
46 Tropomyosin 3	AB062125	<i>TPM3</i>	1q21.2	0.81
47 Tripartite motif-containing 5	NM_033034N	<i>TRIM5</i>	11p15	0.82
48 ZW10 interactor	NM_032997	<i>ZWINT</i>	10q21-q22	0.85
49 ASF1 anti-silencing function 1 homolog A (S. cerevisiae)	NM_014034	<i>ASF1A</i>	6q22.31	1.21
50 Axotrophin	AK022973	<i>AXOT</i>	2q24.2	1.18
51 Carnitine deficiency-associated gene expressed in ventricle 1	AF078932	<i>CDV-1</i>	12q24.13	1.15
52 Kinesin family member 18A	AB062483	<i>DKFZP434G2226</i>	11p14.1	1.33
53 Ets variant gene 5 (ets-related molecule)	NM_004454	<i>ETV5</i>	3q28	1.18
54 Hypothetical protein FLJ20249	NM_015590	<i>FLJ20249</i>	1q22	1.18
55 Histamine N-methyltransferase	NM_006895	<i>HNMT</i>	2q22.1	1.18
56 IDN3 protein	NM_133433	<i>IDN3</i>	5p13.2	1.23
57 Likely ortholog of mouse immediate early response, erythropoietin 4	BC021102	<i>LEREPO4</i>	2q32.2	1.21
58 Neurogenic differentiation 6	NM_022728	<i>NEUROD6</i>	7p15.1	1.21
59 Nuclear factor IB	U70862	<i>NFIB</i>	9p24.1	1.24
60 Oculocerebrorenal syndrome of Lowe	NM_001587	<i>OCRL</i>	Xq25-q26.1	1.21
61 Protein kinase, cAMP-dependent, regulatory, type II, beta	NM_002736	<i>PRKAR2B</i>	7q22	1.17
62 Rho-associated, coiled-coil containing protein kinase 1	NM_005406	<i>ROCK1</i>	18q11.2	1.21
63 Semaphorin 3A	L26081	<i>SEMA3A</i>	7p12.1	1.43
64 C-myc purine-binding transcription factor (PUF), mRNA	XM_070869	<i>siNuclLOC138342</i>	9	1.16
65 Solute carrier family 7 (cationic amino acid transporter)	AB040875	<i>SLC7A11</i>	4q28-q32	1.35
66 Sterol Oacyltransferase 2	AF099031	<i>SOAT2</i>	12q13.13	1.22
67 Translocation protein 1	AB024586	<i>TLOC1</i>	3q26.2-q27	1.19
68 Tetratricopeptide repeat domain 3	NM_003316	<i>TTC3</i>	21q22.2	1.26
69 Zinc finger protein 281	NM_012482	<i>ZNF281</i>	1q32.1	1.27

Discussion

This is the first report on direct gene modulations by RANKL in RANK-positive human osteosarcoma cells, Saos-2. RANK expressed by osteoclasts/osteoclast precursors is recognized as the key molecule involved in osteoclastogenesis and mature osteoclast activation (4). However, its expression is not restricted to the osteoclastic lineage as it is also demonstrated

in other tissues including mammary gland, heart, lung and skeletal muscle (4). Furthermore, recent finding clearly suggested a pivotal role of RANK in bone-associated tumors by presenting RANKL-triggered RANK-positive cell migration (8,9). Moreover, a positive correlation has been reported between constant expressions of RANK with decreased/absent expression of RANKL and a high metastatic phenotype in breast carcinoma (16). We have recently demonstrated

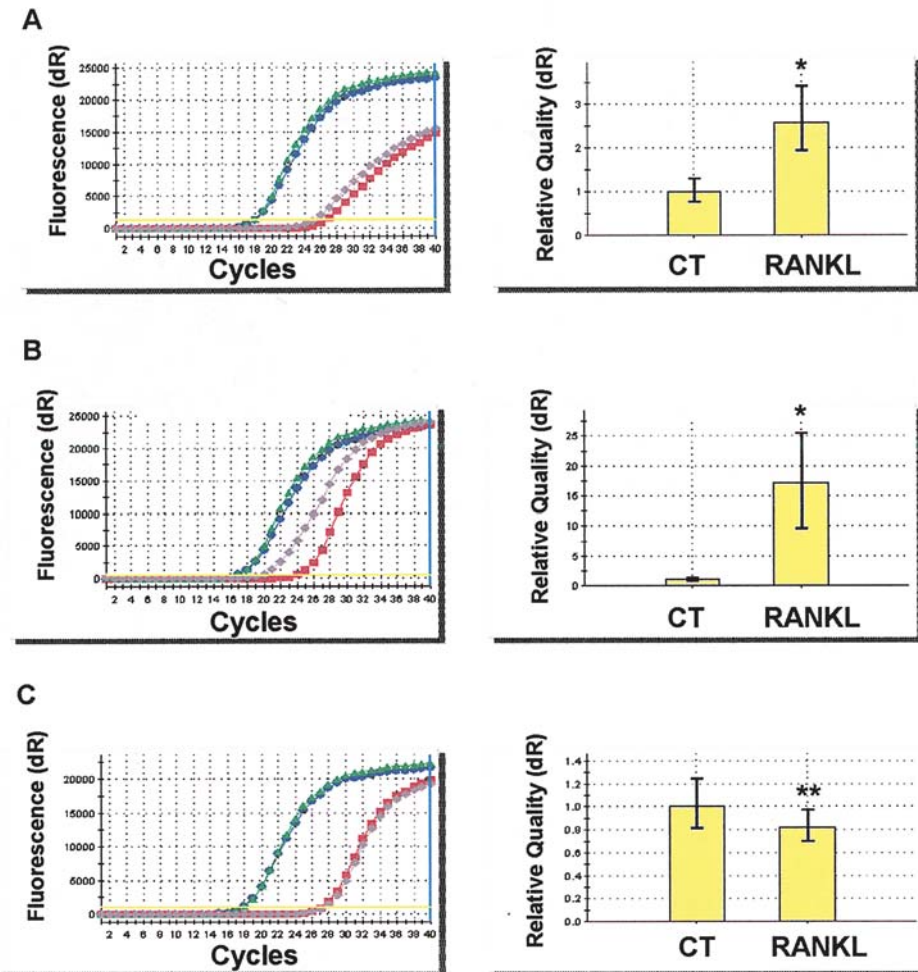


Figure 2. Representative results of quantitative real-time RT-PCR (qRT-PCR). To confirm the results of cDNA microarray analysis, 10% of RANKL-modulated genes were measured by qRT-PCR as described in Materials and methods. (A) ROCK1 (Rho associated, coiled-coil containing protein kinase 1), (B) SEMA3A (Semaphorin 3A) and (C) GDF15 (growth differentiation factor 15). * $p < 0.0001$, ** $p < 0.05$, by Mann-Whitney's U test.

functional RANK expression in several human osteosarcoma cell lines (11). RANK expression in bone-associated tumors is therefore very hot spot of tumor-bone biology.

Alternatively, as bone environment is rich in RANKL, RANKL can bind to RANK expressed on osteosarcoma cells in a paracrine (soluble RANKL) and/or juxtacrine (membrane RANKL) manner. Therefore, RANKL can act as activator of two targets in this tumoral bone environment: one is osteoclasts/osteoclast precursors and the other is RANK-positive osteosarcoma cells. RANKL activates osteoclasts/osteoclast precursors and increases osteoclastic activity leading to disturbed bone remodeling and then releases several tumor-supportive growth factors. This interaction resides between pathologic bone remodeling and osteosarcoma development creates a vicious cycle (17). In high grade osteosarcoma, this vicious cycle should be more accelerated because of its increased RANKL/OPG ratio (7). Such phenomenon has been well documented in osteolytic bone tumors (6-8); however recent findings suggested the importance of osteoclast functions in osteoblastic bone metastasis (18,19). RANK also activates RANK-positive osteosarcoma cells and induce gene modulations. In Saos-2 cells, RANKL up-regulated the expression of genes such as SEMA3A and axotrophin that

exert immunosuppressive activity (20,21). Interestingly, knockout of the SEMA3A gene induces abnormal bone and cartilage development (22). It has been also reported that SEMA3A signaling molecules are in a position to modulate the vascularization of bone, and the innervation of osteoblasts and osteoclasts during bone development and remodeling (23). In this respect, RANKL appears as a deleterious factor thereby facilitating tumor progression. Namely, RANKL might have a propensity for osteosarcoma development by synergistic effect of osteoclasts activity acting as a 'soil' factor in bone environment suggested in other bone-associated tumors (8,9). In addition, RANKL-induced SEMA3A modulation might play, at least in part, the osteoblastic profile of osteosarcoma.

On the contrary, RANKL appears as a protective factor against osteosarcoma development by modulating other genes involved in the cellular metabolism. For instance, RANKL down-regulated genes encoding proteasome 26S and ribophorin I, known to reduce the proteasomal degradation machinery (24,25) and GDF-15 that is associated with early prostate carcinogenesis (26). In addition, RANKL up-regulated NF- κ B which is potentially implicated in cell morphology and susceptibility to nuclear oncogenes (27).

Moreover, except for these direct RANKL-induced gene modulations in RANK-positive osteosarcoma cells, RANKL could be involved as a tumor development protector, as RANKL can act as a potent immune activator by inhibiting dendritic cell apoptosis (28).

Further experiments are needed to determine the balance between pro- and anti-tumor activities of RANKL in osteosarcoma that could provide new therapeutic approaches targeting RANK-positive osteosarcoma.

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